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Neozygites linanensis sp. nov., a fungal pathogen infecting bamboo aphids in southeast China

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Abstract—*Neozygites linanensis* sp. nov. was identified as infecting the aphid *Takecallis taiwanus* collected on bamboo plants, *Chimonobambusa quadrangularis*, in the Lin'an region of southeast China during spring and summer 2014. This is the first record of a *Neozygites* species on *T. taiwanus*. The Chinese *Neozygites* specimen is described, illustrated, and discussed. The phylogenetic relationship among selected *Neozygites* species is evaluated based on the successful sequence of the SSU rDNA gene from the new fungus.

Key words—natural enemies, entomopathogenic fungi, *Entomophthoromycota,* aphid control, *Neozygitaceae*

Introduction

Bamboo plantations are a common forest type in Asia, and bamboo plants supply food, ethnomedicines, and other necessary materials. Moreover, bamboo forests are extremely helpful for carbon sequestration (Li et al. 2013). Worldwide, over 40 aphid species infest bamboos, and many bamboo aphids with robust reproductive and dispersal abilities cause considerable loss of bamboo productivity (Blackman & Eastop 1994). Zhou et al. (2014) recently reported on one entomophthoralean aphid pathogen, *Conidiobolus obscurus*

(I.M. Hall & P.H. Dunn) Remaud. & S. Keller, that infected four bamboo aphid species in the laboratory. However, rarely has information been recorded about the natural enemies and pathogens of bamboo aphids and their interaction in the field. Recently entomopathogenic fungi on bamboo aphids were surveyed in China's Lin'an region, an important bamboo forest area. Mycosed individuals of one widespread bamboo aphid species that frequently infects local bamboo plants, *Takecallis taiwanus* (Blackman & Eastop 1994), were observed during spring and early summer. Initial morphological examination of the infected *T. taiwanus* showed that the entomopathogenic fungus represented *Neozygites*. The only previously reported aphid-pathogenic *Neozygites* species in China is *Neozygites fresenii* (Nowak.) Remaud. & S. Keller, which has been found on *Aphis gossypii* in northern China and on *Myzus persicae* on cabbage in southern China (Zhang et al. 1983, Huang et al. 2008).

Research on *Neozygites* species in natural ecosystems has been limited, particularly in comparison to that reported worldwide for other aphid-specific entomophthoralean species in *Pandora*, *Conidiobolus*, *Entomophthora*, and *Zoophthora* (Barta & Cagáň 2006, Keller 2006). Ben-Ze'ev (1981) reported *Neozygites turbinata* (R.G. Kenneth) Remaud. & S. Keller in Israel on the aphid *Pterochloroides persicae*. *Neozygites lageniformis* (Thaxt.) Remaud. & S. Keller was found on aphids *Myzocallis coryli* on *Corylus avellana* in Chile (Barta & Cagáň 2006). In Switzerland, Keller reported *Neozygites cinarae* S. Keller on *Cinara pilicornis* on *Picea abies* (Keller 1997) and described *Neozygites remaudierei* S. Keller from *Myzocallis coryli* (Keller 2006). Barta (2009) reported eighteen species of aphids on woody plants infected by *N. fresenii* and one species infected by *Neozygites cinarae* in the Mlyňany arboretum in Slovakia. Montalva et al. (2013) discovered a new species, *Neozygites osornensis* Montalva & Barta, on Cinara aphids associated with *Cupressus macrocarpa* and *Austrocedrus chilensis* in Chile. Other aphid-pathogenic species reported in agroecosystems include *Neozygites microlophii* S. Keller on nettle aphids in Switzerland and *Neozygites lecanii* (Zimm.) Ben Ze'ev & R.G. Kenneth on *Lecanium viride* in South East Asia.

Additionally, gene-based research on *Neozygites* species is scarce, which further hinders understanding of the phylogenetic relationships and the status of this genus within the entomophthoroid fungi. Finally, difficulty in obtaining in vitro cultures and its infrequent collection makes *Neozygites* a much-understudied genus (Humber 2012a). The aim of the present work was to identify the *Neozygites* species collected on mycosed *T. taiwanus* populations in bamboo forests through morphological and molecular analyses. For sequencing fungal

DNA from resting spores within host cadavers, we conducted a subcloning procedure that is widely used to diagnose mixed sequences in samples (Pérez-Tris & Bensch 2005). We then used the sequence of the small subunit ribosomal RNA (SSU rRNA) gene obtained from the mycosed *T. taiwanus* specimens to analyze the genetic relationships of other *Neozygites* species.

Materials & methods

Field collection & material processing

The survey of local bamboo–*Chimonobambusa quadrangularis* forests in the suburb of Lin'an city, Zhejiang province, China, was carried out during the spring and summer of 2014. Specimens of dead mycosed adults of *T. taiwanus* attached to bamboo leaves were carefully removed and transferred to a paper bag and then stored in a polystyrene cooler at –20°C (Benjamin et al. 2004). The fungal pathogen (N51) was cultured in vivo by host passage in leaf-containing Petri dishes in the laboratory at 20–24°C. Two types of solid culture medium were tested during our attempts to let the fungal spores eject on the surface of medium and obtain in vitro culture of this fungus: (i) Sabouraud dextrose agar plus yeast extract (SDAY: 4% glucose, 1% peptone, 1% yeast extract, and 1.5% agar) and (ii) SEMA (80% SDAY plus 11.5% egg yolk, 8.5% pure milk) were attempted to cultivate the fungus (Hajek et al. 2012).

Morphological evaluations

The fungus was identified based on morphological characteristics according to Keller (1991, 1997) and Humber (2012a). Semi-permanent slide mounts were prepared in lactophenol-aceto-orcein (LPAO) as described by Keller (1987) and deposited in the Forest Insect and Disease Herbarium, Zhejiang Agricultural and Forestry University, Lin'an, China (ZAFU). Fungal structures were examined with a Zeiss AX10 microscope, photographed with a Zeiss HAL100 digital camera at a magnification of 400×, and measured with the software Zen 2012 (Zeiss). The measurements were based on 50 objects per microstructure. For each microstructure, mean value of measurements, standard error of the mean (± SEM), and maximal and minimal values were calculated, cited below as minimum–mean±SEM–maximum.

The fungal material obtained from *T. taiwanus* in China was microscopically compared with *Neozygites fresenii* attacking *Rhopalosiphum padi* in Poland (where the fungus *N. fresenii* was first described) and *N. fresenii* from Slovakia, Austria (European material was provided by Dr. Marek Barta), and Chile. Two other aphid-pathogenic *Neozygites* species, *N. osornensis* and *N. cinarae*, were obtained during a survey of natural enemies of tree dwelling aphids in southern Chile.

DNA extraction & PCR amplification

Resting spores of the *Neozygites* isolates for DNA extraction were obtained from laboratory-infected *T. taiwanus* bamboo aphids. Fresh mycosed cadavers collected from the field were placed in aphid cohorts in leaf-containing dishes at 20–24°C. After three days of incubation, new cadavers, including black cadavers with resting

Table 1. Morphological comparison of *Neozygites linanesis* with *N. fresenii*, *N. microlophii*, and *N. remaudierei*

Species	Country (source)	Host(s)	Hyphal bodies diam. (µm)	Primary conidia		Capilliconidia		Zygospores	
				L (µm)	W (µm)	L (µm)	W (µm)	L (µm)	W (µm)
Neozygites linanesis	China	*Takecallis taiwanus*	14.5–21	18.8–26.5	14.4–22.8	17.3–29.3	10.6–16.2	21.6–37.2	15.5–26.9
			(10)		(7)		(7)		(2)
Neozygites fresenii	Poland (Bałazy 1993)	3 genera	14–18	18–20	15–18	19–27	11–14	24–49	16–30
	Switzerland (Keller 1991)	3 genera	14–18 (4)	16–24	12–21	16–33	9–17	25–48	17–24
	Slovakia (Barta 2004)	15 genera	—	13.9–25.7	11.9–19.8	15.8–31.7	9.9–19.8	36–46.8	19.8–25.2
Neozygites microlophii	Switzerland (Keller 1991)	*Microlophium carnosum*	17–22	21–30	15–22	19–29	12–19	29–50	17–27
			(5)		(5)				
Neozygites remaudierei	Switzerland (Keller 1991)	*Myzocallis coryli*	17–23	21–25	17–23	24–36	12–18	—	—

Nuclei per structure (in parentheses); L = length; W = width

spores, were found in each dish. Cadavers with resting spores were collected, placed into 1.5 mL tubes (50 cadavers per tube), and stored at 4°C. DNA extraction followed the protocol set forth in the TakaRa (Ver. 5.0) MiniBest universal genomic DNA extraction Kit. To maximize DNA extraction from the resting spores, several 2.5 mm sterile zirconia beads were added into each tube and then vibrated for 1 min, repeated several times during the 56°C water bath for cell lysis. The extracted DNA concentration was quantified by Nanodrop® ND-1000 and maintained at −20°C.

Universal fungal primers NSR 0004-5' (5'-CTGGTTGATTCTGCCAGT-3'; Gargas & DePriest 1996) and SR13 (5'-AATGATCCTTCCGCAGGT-3'; Depress 1993) were used to amplify the nuclear SSU ribosomal RNA gene. PCR was carried out with 30 µl per sample and in duplicates. Each sample contained 2 × PrimeSTAR® Max Premix, 0.12 µM of each primer, sterile distilled water, and 2 µl template (25 ng/µl of fungal DNA isolated). The thermal profile of the 35 cycles of PCR consisted of the initial step at 94°C for 3 min, followed by 35 cycles at 94°C for 15 s, 55°C for 15 s and 72°C for 10 s and the final step at 72 °C for 5 min. PCR products were run in 1% agarose gels with 0.1 µl/mL of ethidium bromide in 1 × TBE buffer at 100 V for 30 min. The PCR products were examined and photographed on a UV light transilluminator.

DNA sequencing & analysis of SSU rDNA sequences

The amplified DNA was sent to TaKaRa Biotechnology (Dalian, China) for TA cloning and sequencing. The target band of ~1.5 kb was recovered, and the DNA fragment was ligated into a pMD18-T vector (TaKaRa, China) after adenine additions. *Escherichia coli* JM109 cells were transformed and spread onto plates of Luria-Bertani agar plus X-Gal, IPTG and Ampicillin. The plates were incubated overnight at 37°C. Three positive colonies (white color) were selected through blue-white screening as replicates. The vector primers M13-47 (5'-CGCCAGGGTTTT CCCAGTCACGAC-3') and RV-M (5'-GAGCGGATAACAATTTCACACAGG-3') were used to amplify the SSU rRNA gene sequence of the *Neozygites* isolate, which was inserted into the pMD18-T plasmid in monoclonal colony PCR. And then the PCR products from each clone were sequenced. Copies of the *Neozygites* sp. (N51) sequences were pairwise compared with other *Neozygites* species online (ClustalW2, the European Bioinformatics Institute, Cambridge, UK). A multiple alignment of sequences was performed with ClustalX 2.0 (Larkin et al., 2007) among SSU rDNA sequences, and a dendrogram was constructed with Seaview 4.0 (Gouy et al. 2010) using the neighbor-joining method with 1000 bootstraps. The sequences obtained in this analysis and from GenBank for other *Neozygites* species such as *N. osornensis* (KC822922), *N. cinarae* (KC822923), *N. turbinata* (KC822924), *N. parvispora* (MacLeod & Carl) Remaud. & S. Keller (AF296760), *N. floridana* (J. Weiser & Muma) Remaud. & S. Keller (AF296758), *N. tanajoae* Delal. et al. (AY233981), and *Neozygites* sp. (AF296759) obtained from GenBank were included in the analysis. Other species infecting aphids on bamboo plants as *Conidiobolus obscurus* (JQ014014) (Zhou et al. 2014) were chosen as outgroup in the phylogenetic tree.

Results

During the survey of natural enemies of the bamboo aphid, mycosed cadavers of *T. taiwanus* were observed from spring to summer 2014. Grey-brown infected aphids were easily recognized hanging on the lower sides of new bamboo leaves. Microscopic observations of the cadavers revealing fungal infection by *Neozygites* represented the first find of mycosed bamboo aphids in Lin'an city, southeast China.

Cultural & morphological observations

No in vitro cultures were successfully isolated from infected individuals, despite attempts to isolate the entomopathogenic fungi using two different solid culture media.

Measurements of taxonomically important structures and comparison with morphologically similar species are presented in Table 1. *Neozygites* obtained from *T. taiwanus* in China was morphologically distinguished from the other aphid *Neozygites* species mainly by higher number of nuclei per hyphal body, primary conidia, and capilliconidia (Plate 1). The sizes of the major morphological microstructures in the Chinese fungus are larger than in *N. fresenii* from Austria and Slovakia, similar in *N. fresenii* from Poland and Chile, and smaller than in *N. osornensis* and *N. cinarae* from Chile (except for a larger capilliconidial diameter in the Chinese *Neozygites* specimens).

Molecular analysis

The fungal SSU rDNA gene from the Chinese *Neozygites* fungus was successfully sequenced and its sequence (KM386989) deposited in the GenBank database (NCBI). Pairwise comparisons of the 1524 bp long aligned SSU rDNA gene sequence showed that the Chinese fungal sequence shares a 92% similarity with *N. turbinata* and a 93% similarity with *N. cinarae* and *N. osornensis*—all species infecting aphid hosts. On the other hand, the Chinese *Neozygites* sequence matched only 73% with *N. tanajoae*, 75% with *N. floridana*, and 77% with *N. parvispora,* all species affecting only mites or thrips. Furthermore, the phylogenetic neighbor-joining SSU rDNA tree separates the bamboo aphid fungus from the clade containing other aphid-associated *Neozygites* species, supporting the fungus found on *T. taiwanus* as an independent species within *Neozygites* (Plate 2).

Taxonomic description

Neozygites linanesis X. Zhou & C. Montalva, **sp. nov.** Plate 1

MycoBank 814659

Differs from *Neozygites fresenii* by its larger hyphal bodies and primary conidia (both containing a greater number of nuclei) and by its smaller zygospores.

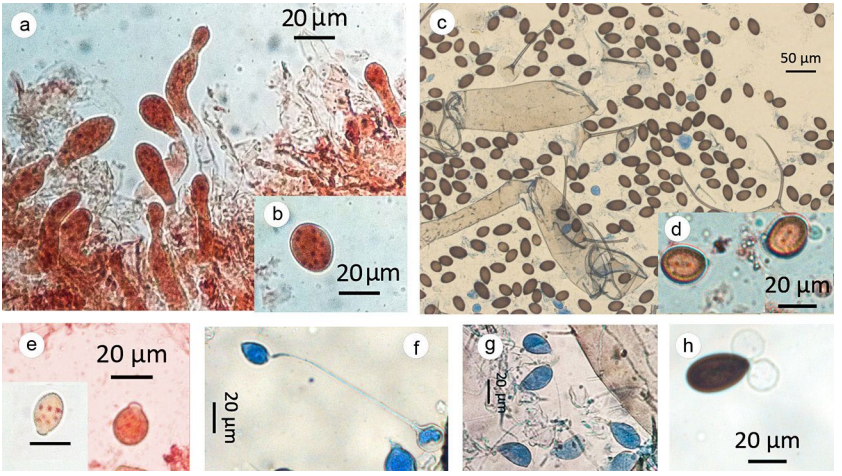

Plate 1. Fungal structures of *Neozygites linanesis* on bamboo aphid *Takecallis taiwanus*. a. Unbranched conidiophores. b. Subspherical hyphal body with visible nuclei. c. Maturing resting spores filling cadaver. d. Resting spores with two visible nuclei. e. Primary conidium. f. A developing capilliconidium on a long slender capillary tube. g. Mature capilliconidia adhered to host leg parts. h. Resting spore developing by conjugation of two spherical hyphal bodies. [Figures a, b, d, e, h stained in lactophenol-aceto-orcein; figures c, f, g in cotton blue.]

Type: China, Zhejiang Province, suburb of Lin'an city, 30°15′49″N 119°44′23″E, from *Takecallis taiwanus* (Takakashi) on *Chimonobambusa quadrangularis* (Fenzl) Makino, 20 May 2014, coll. Xiang Zhou (**Holotype**, ZAFU IP15091101; GenBank KM386989),

Etymology: The specific epithet refers to the name of the type locality.

Hyphal bodies spherical to subspherical, 14.5–17.4±1.6–21 µm in diameter, containing an average of 10 nuclei (1.9 ± 0.6 µm diam.); primary conidia spherical, 18.8–22.9±1.8–26.5 × 14.4–17.6±1.9–22.8 µm with a distinct cylindrical papilla, containing an average of 7 nuclei; capilliconidia forming from primary conidia, almond-shaped, 17.3–23.3±2.3–29.3 × 10.6–13.6±1.6–16.2 µm, containing an average of 7 nuclei, borne on a long slender capillary tube 66.5 ± 15.8 µm; zygospores broadly ellipsoidal, thick-walled, with a black, smooth episporium, 21.6–28.1±3.7–37.2 × 15.5–19.2±2.3–26.9 µm. Rhizoids not observed.

Observed in spring and summer 2014 in just one locality in China.

Discussion

The genus *Neozygites,* which differs from the other entomophthoroid fungal species by the nuclear structure and behaviour during mitosis (Butt & Humber 1989), currently comprises 19 entomopathogenic fungal species that

may eventually be split into two genera differentiated by their host affinities and resting spore morphologies (Humber 2012b). The nine aphid-pathogenic species (Keller 2006, Montalva et al. 2014a) have smooth ovoid resting spores, and none of these species (including the type species, *N. fresenii*) has been grown in vitro; the remaining 10 species infect primarily mites (but include some species on non-aphid insect hosts) and produce rough globose zygospores; all in vitro cultures of *Neozygites* species have been isolated from mites.

From the morphological point of view, *N. fresenii* and *N. linanesis* are closely related. *Neozygites fresenii* is a widely distributed fungal pathogen of aphids (Milner & Holdom 1986, Hatting et al. 1999, Barta & Cagáň 2006, Scorsetti et al. 2007). It was named and described as *Empusa fresenii* by Nowakowski (1883), who first discovered the fungus in naturally infected aphids collected in Poland. This is the first described member of the *Neozygites* genus. While both fungi are pathogenic to aphids, *N. linanesis* can be distinguished from *N. fresenii* mainly by its greater average number of nuclei per hyphal body and primary conidium. Also, the hyphal bodies and primary spores in *N. linanesis* are somewhat larger than those in *N. fresenii* recorded in the literature from Poland, Switzerland, and Slovakia, while its zygospores are slightly small compared those recorded for *N. fresenii* (Keller 1991, Bałazy 1993, Barta 2004). Compared to other aphid-pathogenic *Neozygites* species, all the recorded dimensions for microstructures in *N. microlophii* and *N. remaudierei* are larger than those in *N. linanesis* (Keller 1991, 2006).

Unfortunately, there are no DNA sequences from *N. fresenii* available for molecular comparisons with other *Neozygites* species. Nonetheless, SSU rDNA gene analysis from *N. linanesis* and the other aphid-pathogenic *Neozygites* species showed clearly that *N. linanesis* is separated in a different clade from *N. cinarae*, *N. turbinata*, and *N. osornensis* (Plate 2); this analysis, when combined with the morphological differences, suggests that *N. linanesis* is a distinct species within *Neozygites*.

Moreover, the molecular differences between aphid- and mite-/thrip-pathogenic *Neozygites* species are consistent with the proposal by Humber (2012b). SSU rDNA gene-based analysis successfully separated *N. linanesis* from a morphologically similar *Neozygites* species, thus supporting the morphological observations made in this study. This gene has been previously used to separate *N. tanajoae* from the morphologically similar *N. floridana* (Delalibera & Humber 2004) as well as *N. osornensis* from *N. turbinata* and *N. cinarae* (Montalva et al. 2014b). We regard the SSU rDNA gene as a valid tool for identifying *Neozygites* species.

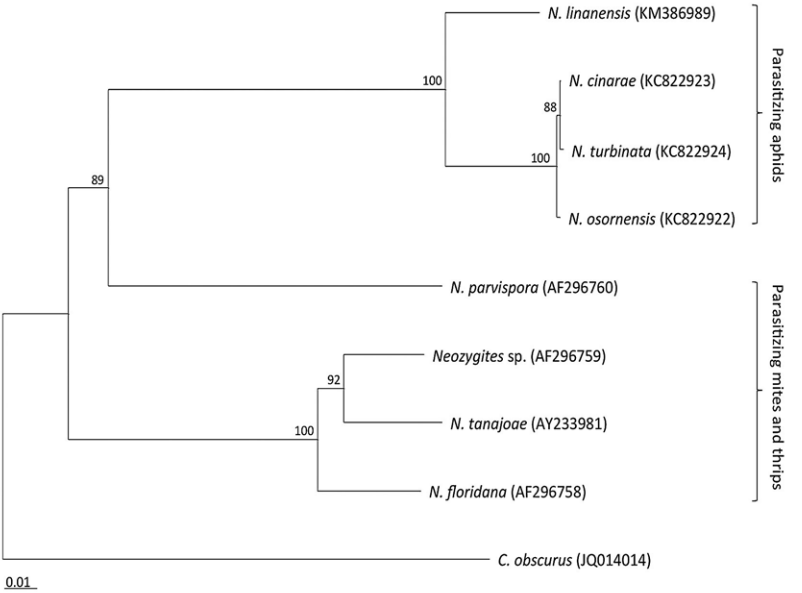

Plate 2. Dendrogram showing the relationships among SSU rDNA sequences of *Neozygites linanesis* isolated from bamboo aphid *Takecallis taiwanus* and other DNA sequences of *Neozygites* species published in GenBank. *Conidiobolus obscurus* was used as outgroup and the numbers on each branch indicate bootstrap values (1000 replicates).

In this work we have described a new *Neozygites* species that can infect *Takecallis* aphids and observed the spread of *N. linanensis* in host populations during the aphid infestation period. *Neozygites linanesis,* which appears to be a natural control agent of *T. taiwanus* in bamboo forests in China, represents the first record of a *Neozygites* species associated with *T. taiwanus* populations. Our 2014 surveys also revealed that *N. linanesis* infects other aphid species in China, such as *Metamacropodaphis bambusisucta.* The identification of *N. linanesis* and the sequencing of its SSU rDNA gene have thereby added to the knowledge regarding natural fungal enemies of bamboo aphids.

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